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am the translator of the documents attached and I state that the following is a true translation to the best of my knowledge and belief of International Patent Application No. PCT/EP2004/011512 filed on October 13, 2004.

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(Signature of translator)

Recombinant vaccines and use thereof

The present invention relates to fusion molecules of antigens, to the nucleic acids coding therefor and to the use of such fusion molecules and nucleic acids. The invention relates in particular to fusion molecules which comprise an antigen and the transmembrane region and cytoplasmic region of an MHC molecule or the cytoplasmic region of an MHC molecule or of a SNARE molecule.

Fusion molecules of the invention can be used for a large number of applications, including in methods for inducing an immune response in a mammal.

Antigen-specific T cell reactions are elicited by antigenic peptides which are bound to the binding groove of glycoproteins of the major histocompatibility complex (MHC), as part of the mechanism of the immune system in which foreign antigens are identified and a response to them is induced. The bound antigenic peptides interact with T cell receptors and thus modulate an immune response. The antigenic peptides are non-covalently bound to certain "binding pockets" formed by polymorphic residues of the binding groove of the MHC protein.

MHC class II molecules are heterodimeric glycoproteins consisting of α and β chains. The α1 and β1 domains of these molecules fold together and form a peptide-binding groove. Antigenic peptides bind to the MHC molecule through interaction between anchor amino acids on the peptide and α1 and β1 domains. The crystal structure of the human class II HLA DR1 complex with an influenza virus peptide shows that the N and C terminal ends of the bound peptide extend out of the binding groove, so that the C terminus of the peptide lies near to the N terminus of the β chain [Brown, J.H. et al., 1993, Nature 364:33-39; Stern, L.J. et al., 1994,

Nature 368:215-221]. MHC class I molecules have different domain organizations than MHC class II molecules but generally a similar structure with a peptide-binding site or groove which is remote from the membrane domains [cf. for example Rudensky, A.Y. et al., 1991, Nature 353:622-627].

The initial step in the presentation of a foreign protein antigen is binding of the native antigen to an antigen-presenting cell (APC). After binding to APCs, antigens penetrate into the cells, either by phagocytosis, receptor-mediated endocytosis or pinocytosis. Such internalized antigens are located in intracellular membrane-bound vesicles called endosomes. Following endosome-lysosome fusion, the antigens are processed to small peptides by cellular proteases present in the lysosomes. The peptides associate with the α and β chains of MHC class II molecules within these lysosomes. These MHC class II molecules, which had previously been synthesized in the rough endoplasmic reticulum, are transported sequentially to the Golgi complexes and then to the lysosomal compartment. The peptide-MHC complex is presented on the surface of APCs for T- and B-cell activation. Therefore, the accessibility of proteolytic processing sites in the antigen, the stability of the resulting peptides in the lysosomes and the affinities of the peptides for MHC molecules are determining factors for the immunogenicity of a specific epitope.

Recombinant vaccines have particular importance in human and veterinary medicine as agents and medicaments for the prophylaxis and therapy of infectious diseases and cancers. The aim of vaccination with a recombinant vaccine is to induce a specific immune response to a defined antigen, which response has preventive or therapeutic activity against defined diseases.

A factor which is essential for the efficacy of a recombinant vaccine is optimal stimulation of T lymphocytes of the immunized organism. Thus, a number of animal-experimental investigations demonstrates that both optimal stimulation of $CD8^+$ and $CD4^+$ lymphocytes is necessary for effective immunotherapy of tumors. The known major types of recombinant vaccines are based on recombinant proteins, synthetic peptide fragments, recombinant viruses and nucleic acid vaccines based on DNA or RNA. In recent years, vaccines based on DNA and RNA nucleic acids have become increasingly important. However, only very poor or even no stimulation of $CD4^+$ lymphocytes can be achieved with recombinant vaccines based on nucleic acids for very many aims, inter alia tumour antigens. For this reason, a number of genetic modifications has been developed with the intention of increasing the immunogenicity of recombinant vaccines. Various methods have been tested in this connection to date, inter alia heterogenization of immunogens by altering the primary sequence or by fusion to foreign epitopes, e.g. from bacteria or viruses [Lowenadler, B. et al., 1990, Eur. J. Immunol. 20: 1541-45; Clarke, B.E. et al., 1987, Nature 330: 381-84] and preparation of chimeric products consisting of the actual antigen and immunomodulatory proteins such as cytokines [Ruckert, R. et al., 1998, Eur. J. Immunol. 28: 3312-20; Harvill, E. T., J. M. Fleming, and S. L. Morrison, 1996, J. Immunol. 157: 3165-70]. Although vaccines based on heterogenization induce enhanced immune responses, they have the great disadvantage that immunostimulation against the foreign epitope predominates and that immune responses against the actual vaccine target remain only moderate in some cases.

A further attractive possibility is fusion to sequences of proteins intended to permit translocation of the protein into degrading cell compartments. However, it is now known that these modifications lead to only a

moderate improvement in stimulation of $CD4^+$ lymphocytes and to scarcely any enhancement of $CD8^+$ immune responses [Wu, T.C. et al., 1995, Proc. Natl. Acad. Sci. U.S.A. 92: 11671-11675; Bonini, C. et al., 2001, J. Immunol. 166: 5250-57, Su, Z. et al., 2002, Cancer Res. 62: 5041-5048].

It would thus be desirable for vaccines which distinctly increase antigen presentation and thus immunogenicity in relation to a particular antigen to be available. It would further be desirable for it to be possible to modify vaccines systematically in such a way that a maximum immune response by $CD4^+$ and $CD8^+$ lymphocytes results, without the need to introduce foreign epitopes.

This object is achieved according to the invention by the subject matter of the claims.

It has been possible to establish according to the invention that fusion molecules comprising antigen molecules and parts of histocompatibility antigens show, when used as vaccines, an immunogenicity which is increased >100-fold compared with the unmodified antigens, and that surprisingly both immune responses of $CD4^+$ and $CD8^+$ T lymphocytes are increased in a manner not previously described.

The present invention relates in general to fusion molecules of antigen molecules and to the use of such fusion molecules.

In one aspect, the invention relates to a fusion molecule which comprises an antigen and the cytoplasmic region of a chain of an MHC molecule, or an antigen, a transmembrane region and the cytoplasmic region of a chain of an MHC molecule. It is preferred for both the transmembrane region and the cytoplasmic region to be

derived from a MHC molecule. In addition, the fusion molecule preferably comprises no MHC binding domain.

The invention further relates to a fusion molecule which comprises an antigen and a chain of an MHC molecule or a part thereof, where the part comprises at least the transmembrane region and the cytoplasmic region of the chain of the MHC molecule. The part of the chain of an MHC molecule preferably does not comprise the MHC binding domain or parts thereof. There is thus provided in particular a fusion molecule which comprises an antigen and a part of a chain of an MHC molecule, which part corresponds essentially to the sequence of the transmembrane region connected to the cytoplasmic region of an MHC molecule, where the expression "transmembrane region connected to the cytoplasmic region" relates to the segment of a chain of an MHC molecule which starts with the N-terminal end of the transmembrane region and terminates with the C-terminal end of the cytoplasmic region, in particular the C-terminal end of the complete chain of the MHC molecule. In this embodiment, the connection of the transmembrane region to the cytoplasmic region corresponds to the naturally occurring connection between these regions.

The invention further provides a fusion molecule which comprises an antigen and a chain of an MHC molecule or a part thereof, where the part essentially lacks the complete N-terminal extracellular domains of the MHC molecule.

In a particularly preferred embodiment, the fusion molecules of the invention consist of a fusion of an antigen, where appropriate with a leader sequence at its N-terminal end, to a transmembrane region, preferably a transmembrane region of a chain of an MHC molecule, at the C-terminal end of the antigen and of a

cytoplasmic region of a chain of an MHC molecule at the C-terminal end of the transmembrane region.

In a particularly preferred embodiment, the fusion molecules of the invention comprise a leader sequence, preferably a peptide sequence having the properties of a secretion signal which is able in particular to control translocation of a protein or peptide through a membrane. It is possible to use as leader sequence the secretion signal of any type I transmembrane protein, where the expression "type I transmembrane protein" relates to those transmembrane proteins whose C terminus is located in the cytoplasm. In a particular embodiment, the leader sequence is derived from a chain of an MHC molecule. The leader sequence is preferably located at the N-terminal end of the fusion molecules of the invention.

In a further aspect, the invention relates to a fusion molecule where essentially the complete N-terminal extracellular domains of an MHC molecule are replaced by an antigen having a leader sequence at its N-terminal end.

It is preferred in a fusion molecule of the invention for the antigen to be covalently connected at its N terminus to the C terminus of a leader sequence, and the C terminus of the antigen molecule is connected to the N terminus of the transmembrane region which in turn is connected at the C terminus to the N terminus of the cytoplasmic region of an MHC molecule.

Thus, the fusion molecule of the invention preferably has the following arrangement: N terminus leader sequence/antigen/transmembrane region/cytoplasmic region C terminus.

In a particularly preferred embodiment, the fusion

molecule of the invention consists essentially of the leader sequence, the antigen, the transmembrane region and the cytoplasmic region.

In a particularly preferred embodiment, the antigen is a peptide, polypeptide or protein, and the fusion molecule of the invention is a protein or polypeptide.

In one embodiment, a plurality of antigens which may be identical or different are present in the fusion molecule of the invention, i.e. at least 2, preferably 2 to 10, more preferably 2 to 5, even more preferably 2 to 3, in particular 2, antigens. These multiply coupled antigens may be present separate from one another or in series one after the other, where appropriate separated by a linker, as tandem constructs. It is preferred for an immune response to various antigens to be induced thereby on administration.

The antigen may be complete or truncated, i.e. it contains only a part of the natural protein or polypeptide which serves as antigen.

The leader sequence and/or the transmembrane region of the fusion molecules of the invention are preferably derived from MHC molecules, in particular of class I or II. It is more preferred for the leader sequence and/or the transmembrane region and/or the cytoplasmic region of the fusion molecules of the invention to be derived from MHC molecules, in particular of class I or II.

It is also possible according to the invention for one or more, preferably flexible, linker sequences (connecting sequences) to be present in the fusion molecule, possibly being located between the leader sequence and the antigen, between the antigen and the transmembrane region and/or between the transmembrane region and the cytoplasmic region. It is preferred

according to the invention for a linker sequence to comprise about 7 to 20 amino acids, more preferably about 8 to 16 amino acids, and in particular about 8 to 12 amino acids.

The linker sequence in fusion molecules of the invention is preferably flexible and thus does not hold the peptide connected therewith in a single, unwanted conformation. The linker preferably comprises in particular amino acids having small side chains, such as glycine, alanine and serine, in order to make flexibility possible. The linker sequence preferably comprises no proline residue, which might inhibit the flexibility.

In a further embodiment, the leader sequence, the antigen, the transmembrane region and/or the cytoplasmic region are connected together directly without a linker.

The leader sequence preferably has the sequence shown in SEQ ID NO: 2 or a sequence derived therefrom, or is encoded by the sequence shown in SEQ ID NO: 1 or a sequence derived therefrom. The transmembrane-cytoplasmic region preferably has the sequence shown in SEQ ID NO: 4 or 6 or a sequence derived therefrom, or is encoded by the sequence shown in SEQ ID NO: 3 or 5 or a sequence derived therefrom.

In further preferred embodiments, the transmembrane-cytoplasmic or the exclusively cytoplasmic region is derived from sequence-related MHC molecules (inter alia HLA-A, HLA-B, HLA-C, HLA-E, HLA-F, HLA-DRa, HLA-DRb, HLA-DQa, HLA-DQb, HLA-DPa, HLA-DPb, CD1a, CD1b, CD1c). Preferred transmembrane-cytoplasmic regions have a sequence selected from the group consisting of the sequences depicted in SEQ ID NO: 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, and sequences

derived therefrom. In further embodiments, the exclusively cytoplasmic regions have a sequence selected from the group consisting of the sequences depicted in SEQ ID NO: 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, and sequences derived therefrom. Further embodiments also provide for the use of varied sequences, e.g. modified or orthologous sequences from different organisms. Sequences particularly preferred in this connection are those having at the C-terminal end a homology of more than 60% with the sequences shown in SEQ ID NO: 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42.

In a particularly preferred embodiment, the fusion molecule of the invention comprises the amino acid sequence shown in SEQ ID NO: 12 or 14, or a sequence derived therefrom.

The invention further relates to a fusion molecule comprising an antigen and a SNARE protein (in particular Cis-golgi SNARE p28, VTI1b, membrin, pallidin, syntaxin-5, syntaxin-6, syntaxin-7, syntaxin-8, syntaxin-10, syntaxin-10a, syntaxin-11, syntaxin-12, syntaxin-17, VAMP-2, VAMP-3, VAMP-4, VAMP-7, VAMP8, VTI1-a-beta, XP350893, LIP5 (SEQ ID NO: 43-63)) or a sequence which comprises one or more SNARE motifs. Targeted transport of the antigen into a defined compartment (e.g. lysosomes and endosomes) is possible by fusing an antigen to a SNARE protein or a SNARE motif (preferably at the C terminus of the SNARE protein or motif). A further possibility with such a targeted transport is for immunogenic epitopes of the antigen to be generated and presented in a compartment, as can be established experimentally.

SNARE proteins are membrane-associated proteins whose common feature is the SNARE motif which comprises 60-70 amino acids. SNARE proteins are functionally involved

in the transport and fusion of vesicles in the cell. Eukaryotic organisms have a large number of different SNARE proteins which are associated with different vesicle membranes in the cell (inter alia endosomal, lysosomal, Golgi, plasma membranes). The cytoplasmic regions of the SNARE proteins have a dual function. Firstly, they serve as trafficking signals (address labels) which specify the destination of the protein and of the associated membrane. Secondly, the domains may contribute through hetero- and homoassociation (joining together) to fusion of different vesicles (e.g. endosomes with lysosomes).

It is also possible according to the invention for the SNARE-antigen fusion molecules to comprise linker sequences between the SNARE portion and the antigen portion. Also included in relation to the antigen and the linker sequence of the SNARE-antigen fusion molecules are all the embodiments described above. A linker in relation to the SNARE-antigen fusion molecules preferably comprises 80-120 amino acids. In a particular embodiment, the linker comprises a transmembrane region. The invention thus relates to fusion molecules which comprise a SNARE protein or a SNARE motif fused to an antigen or a transmembrane region and an antigen. Such fusion molecules are shown for example in Figure 7.

In a further aspect, the invention relates to nucleic acids and derivatives thereof which code for the fusion molecules described above and are preferably able to express these fusion molecules. The term "nucleic acid" hereinafter also includes derivatives thereof.

In a particularly preferred embodiment, the nucleic acid which codes for a fusion molecule of the invention comprises the nucleic acid sequence shown in SEQ ID NO: 11 or 13, or a sequence derived therefrom.

The invention also relates to host cells which comprise a nucleic acid of the invention.

The host cell may moreover comprise a nucleic acid which codes for an HLA molecule. In one embodiment, the host cell expresses the HLA molecule endogenously. In a further embodiment, the host cell expresses the HLA molecule recombinantly. The host cell is preferably non-proliferative. In a preferred embodiment, the host cell is an antigen-presenting cell, in particular a dendritic cell, a monocyte or a macrophage.

In a further aspect, the invention relates to a pharmaceutical composition, in particular a vaccine, which comprises one or more of the fusion molecules of the invention and/or one or more of the nucleic acids coding therefor and/or one or more of the host cells of the invention.

In a further aspect, the invention provides a method for increasing the amount of MHC/peptide complexes in a cell, where the method comprises the provision of a fusion molecule of the invention or of a nucleic acid coding therefor for the cell. The cell is preferably present in a living creature, and the method comprises administering a fusion molecule of the invention or a nucleic acid coding therefor to the living creature. In a preferred embodiment, the cell is an antigen-presenting cell, in particular a dendritic cell, a monocyte or a macrophage.

In a further aspect, the invention provides a method for increasing the presentation of cell surface molecules on cells which are able to present antigens (such as B cells and macrophages, generally called "APC"). The antigen-presenting activity of such cells is enhanced by providing a fusion molecule of the

invention or a nucleic acid coding therefor for the cells. Such an enhancement of the antigen-presenting activity in turn preferably enhances the primary activation of T cells, in particular of $CD4^+$ and $CD8^+$ lymphocytes, which respond to the antigen. The cell is preferably present in a living creature, and the method comprises administering a fusion molecule of the invention or a nucleic acid coding therefor to the living creature.

In a further aspect, the invention provides a method for inducing an immune response in a living creature, where the method comprises the adminisration of a fusion molecule of the invention and/or a nucleic acid coding therefor and/or a host cell of the invention to the living creature.

In a further aspect, the invention provides a method for stimulating or activating T cells, especially $CD4^+$ and $CD8^+$ lymphocytes, in vitro or in a living creature, in particular a patient, where the method comprises the provision for the T cells or administration to the living creature of a fusion molecule of the invention and/or a nucleic acid coding therefor and/or a host cell of the invention. Such a stimulation or activation is preferably expressed in an expansion, cytotoxic reactivity and/or cytokine release by the T cells.

A further aspect provides a method for the treatment, vaccination or immunization of a living creature, where the method comprises the administration a fusion molecule of the invention and/or a nucleic acid coding therefor and/or a host cell of the invention to the living creature. In this connection, the antigens employed in the fusion molecule of the invention or the nucleic acid coding therefor are in particular those which are known to be effective without the alteration according to the invention for the intended treatment,

vaccination or immunization.

The methods described above are particularly suitable for a treatment or prophylaxis of infectious diseases caused for example by bacteria or viruses. In particular embodiments, the antigen used according to the invention is derived from an infectious agent such as hepatitis A, B, C, HIV, mycobacteria, malaria pathogens, SARS pathogens, herpesvirus, influenzavirus, poliovirus or from bacterial pathogens such as chlamydia and mycobacteria. A particularly beneficial application of the present invention is in cancer immunotherapy or vaccination, where there is in particular enhancement of activation of tumor antigen-reactive T cells, thus improving the prospects for T-cell immunotherapy or vaccination against tumor cells.

In specific embodiments, the antigen used according to the invention is selected from the group consisting of the following antigens: p53, preferably encoded by the sequence shown in SEQ ID NO: 66, ART-4, BAGE, ss-catenin/m, Bcr-abL CAMEL, CAP-1, CASP-8, CDC27/m, CDK4/m, CEA, CLAUDIN-12, c-MYC, CT, Cyp-B, DAM, ELF2M, ETV6-AML1, G250, GAGE, GnT-V, Gap100, HAGE, HER-2/neu, HPV-E7, HPV-E6, HAST-2, hTERT (or hTRT), LAGE, LDLR/FUT, MAGE-A, preferably MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11 or MAGE-A12, MAGE-B, MAGE-C, MART-1/melan-A, MC1R, myosin/m, MUC1, MUM-1, -2, -3, NA88-A, NF1, NY-ESO-1, NY-BR-1, p190 minor bcr-abL Pml/RARa, PRAME, proteinase-3, PSA, PSM, RAGE, RU1 or RU2, SAGE, SART-1 or SART-3, SCGB3A2, SCP1, SCP2, SCP3, SSX, SURVIVIN, TEL/AML1, TPI/m, TRP-1, TRP-2, TRP-2/INT2, TPTE and WT, preferably WT-1, in particular encoded by the sequence shown in SEQ ID NO: 65.

Detailed description of the invention

The terms "domain" or "region" relate to a particular part of an amino acid sequence which can preferably be connected to a specific function or structure. For example, the α and β polypeptides of an MHC class II molecule have two domains, α1, α2, and β1, β2, respectively, a transmembrane region and a cytoplasmic region. In a similar manner, the α chain of MHC class I molecules has three domains, α1, α2 and α3, a transmembrane region and a cytoplasmic region.

In one embodiment, the complete domain or region is included in a selection of the sequence of a particular domain or region for deletion or incorporation into a fusion molecule of the invention. In order to ensure this, the sequence of the relevant domain or region can be extended in order to comprise parts of a linker or even parts of the adjacent domain or region. The term "essentially" in relation to a domain or region is to be understood in this sense.

The term "transmembrane region" relates to the part of a protein which essentially accounts for the portion present in a cellular membrane and preferably serves to anchor the protein in the membrane. A transmembrane region is preferably according to the invention an amino acid sequence which spans the membrane once. However, it is also possible in certain embodiments to use a transmembrane region which spans the membrane more than once. The transmembrane region will generally have 15-25 preferably hydrophobic uncharged amino acids which assume for example an α-helical conformation. The transmembrane region is preferably derived from a protein selected from the group consisting of MHC molecules, immunoglobulins, CD4, CD8, the CD3 ζ chain, the CD3 γ chain, the CD3 δ chain and the CD3 ε chain.

The transmembrane region typically consists in the case of the α and β chains of the MHC class II molecule of about 20 hydrophobic amino acids which are connected to the carboxy-terminal end of the antigen. These residues allow the protein to span the membrane. The transmembrane region terminates with about 6-32 residues which comprise the cytoplasmic tail at the carboxy-terminal end of each of these chains. It has been shown that these transmembrane and cytoplasmic regions can be replaced by sequences which signal a GPI binding, and that the chimeric GPI-anchored class II molecules are membrane-bound (Wettstein, D.A., J.J. Boniface, P.A. Reay, H. Schild and M.M. Davis, 1991, J. Exp. Med. 174: 219-228). Such embodiments are encompassed by the term "transmembrane region" according to the invention. GPI-bound membrane anchor domains have been defined in a number of proteins, including decay-accelerating factor (DAF), CD59 and human placental alkaline phosphatase (HPAP) (Wettstein, D.A., J.J. et al., 1991, J. Exp. Med. 174:219-228). For example, the 38 carboxy-terminal amino acids of HPAP are sufficient for functioning as signal sequence for GPI binding. If the DNA sequence coding for this domain is connected to a secreted molecule, such as the soluble part of the MHC class II α or β chain, there is formation of a membrane-bound chimeric molecule (Wettstein, D.A. et al., 1991, J. Exp. Med. 174: 219-228), and a method of this type can be employed to anchor fusion molecules of the invention to a cell membrane.

The term "major histocompatibility complex" and the abbreviation "MHC" relate to a complex of genes which occurs in all vertebrates. The function of MHC proteins or molecules in signaling between lymphocytes and antigen-presenting cells in normal immune responses involves them binding peptides and presenting them for possible recognition by T-cell receptors (TCR). MHC

molecules bind peptides in an intracellular processing compartment and present these peptides on the surface of antigen-presenting cells to T cells. The human MHC region, also referred to as HLA, is located on chromosome 6 and comprises the class I region and the class II region.

The term "MHC class I" or "class I" relates to the major histocompatibility complex class I proteins or genes. Within the human MHC class I region there are the HLA-A, HLA-B, HLA-C, HLA-E, HLA-F, CD1a, CD1b and CD1c subregions.

The class I α chains are glycoproteins having a molecular weight of about 44 kDa. The polypeptide chain has a length of somewhat more than 350 amino acid residues. It can be divided into three functional regions: an external, a transmembrane and a cytoplasmic region. The external region has a length of 283 amino acid residues and is divided into three domains, α1, α2 and α3. The domains and regions are usually encoded by separate exons of the class I gene. The transmembrane region spans the lipid bilayer of the plasma membrane. It consists of 23 usually hydrophobic amino acid residues which are arranged in an α helix. The cytoplasmic region, i.e. the part which faces the cytoplasm and which is connected to the transmembrane region, typically has a length of 32 amino acid residues and is able to interact with the elements of the cytoskeleton. The α chain interacts with β2-microglobulin and thus forms α-β2 dimers on the cell surface.

The term "MHC class II" or "class II" relates to the major histocompatibility complex class II proteins or genes. Within the human MHC class II region there are the DP, DQ and DR subregions for class II α chain genes and β chain genes (i.e. DPα, DPβ, DQα, DQβ, DRα and DRβ).

Class II molecules are heterodimers each consisting of an α chain and a β chain. Both chains are glycoproteins having a molecular weight of 31-34 kDa (α) or 26-29 kDA (β). The total length of the α chains varies from 229 to 233 amino acid residues, and that of the β chains from 225 to 238 residues. Both α and β chains consist of an external region, a connecting peptide, a transmembrane region and a cytoplasmic tail. The external region consists of two domains, α1 and α2 or β1 and β2. The connecting peptide is respectively 13 and 9 residues long in α and β chains. It connects the two domains to the transmembrane region which consists of 23 amino acid residues both in α chains and in β chains. The length of the cytoplasmic region, i.e. the part which faces the cytoplasm and which is connected to the transmembrane region, varies from 3 to 16 residues in α chains and from 8 to 20 residues in β chains.

The term "chain of an MHC molecule" relates according to the invention to the α chain of an MHC class I molecule or to the α and β chains of an MHC class II molecule. The α chains of an MHC class I molecule, from which the fusion molecules of the invention can be derived, comprise the HLA-A, -B and -C α chains. The α chains of an MHC class II molecule, from which the fusion molecules of the invention may be derived, comprise HLA-DR, -DP and -DQ α chains, in particular HLA-DR1, HLA-DR2, HLA-DR4, HLA-DQ1, HLA-DQ2 and HLA-DQ8 α chains and, in particular, α chains encoded by DRA*0101, DRA*0102, DQA1*0301 or DQA1*0501 alleles. The β chains of an MHC class II molecule, from which the fusion molecules of the invention may be derived, comprise HLA-DR, -DP and -DQ β chains, in particular HLA-DR1, HLA-DR2, HLA-DR4, HLA-DQ1, HLA-DQ2 and HLA-DQ8 β chains and, in particular, β chains encoded by DRB1*01, DRB1*15, DRB1*16, DRB5*01, DQB1*03 and DQB1*02 alleles.

The term "MHC binding domain" relates to the "MHC class I binding domain" and "MHC class II binding domain".

The term "MHC class I binding domain" relates to the region of an MHC class I molecule or of an MHC class I chain which is necessary for binding to an antigenic peptide. An MHC class I binding domain is formed mainly by the $\alpha1$ and $\alpha2$ domains of the MHC class I α chain. Although the $\alpha3$ domain of the α chain and $\beta2$-microglobulin do not represent essential parts of the binding domain, they are presumably important for stabilizing the overall structure of the MHC class I molecule and therefore the term "MHC class I binding domain" preferably includes these regions. An MHC class I binding domain can also be essentially defined as the extracellular domain of an MHC class I molecule, distinguishing it from the transmembrane and cytoplasmic regions.

The term "MHC class II binding domain" relates to the region of an MHC class II molecule or of an MHC class II chain which is necessary for binding to an antigenic peptide. An MHC class II binding domain is mainly formed by the $\alpha1$ and $\beta1$ domains of the MHC class II α and β chains. The $\alpha2$ and $\beta2$ domains of these proteins are, however, presumably also important for stabilizing the overall structure of the MHC binding groove, and therefore the term "MHC class II binding domain" according to the invention preferably includes these regions. An MHC class II binding domain can also be defined essentially as the extracellular domain of an MHC class II molecule, distinguishing it from the transmembrane and cytoplasmic domains.

The exact number of amino acids in the various MHC molecule domains or regions varies depending on the mammalian species and between gene classes within a

species. When selecting the amino acid sequence of a particular domain or region, maintenance of the function of the domain or region is much more important than the exact structural definition, which is based on the number of amino acids. The skilled worker is also aware that the function can also be maintained if rather less than the complete amino acid sequence of the selected domain or region is used.

The term "antigen" relates to an agent against which an immune response is to be generated. The term "antigen" includes in particular proteins, peptides, polysaccharides, nucleic acids, especially RNA and DNA, and nucleotides. The term "antigen" also includes derivatized antigens as secondary substance which becomes antigenic - and sensitizing - only through transformation (e.g. intermediately in the molecule, by completion with body protein), and conjugated antigens which, through artificial incorporation of atomic groups (e.g. isocyanates, diazonium salts), display a new constitutive specificity. In a preferred embodiment, the antigen is a tumor antigen, i.e. a constituent of cancer cells which may be derived from the cytoplasm, the cell surface and the cell nucleus, in particular those antigens which are produced, preferably in large quantity, intracellularly or as surface antigens on tumor cells. Examples are carcinoembryonic antigen, α1-fetoprotein, isoferritin and fetal sulfoglycoprotein, α2-H-ferroprotein and γ-fetoprotein and various viral tumor antigens. In a further embodiment, the antigen is a viral antigen such as viral ribonucleoproteins or envelope proteins. In particular, the antigen or peptides thereof should be presented by MHC molecules and thus be able to modulate, in particular, activate, cells of the immune system, preferably $CD4^+$ and $CD8^+$ lymphocytes, in particular by modulating the activity of a T-cell receptor, and thus preferably induce T cell

proliferation.

The term "MHC/peptide complex" relates to a non-covalent complex of the binding domain of an MHC class I or MHC class II molecule and of an MHC class I or MHC class II binding peptide.

The term "MHC binding peptide" or "binding peptide" relates to a peptide which binds to an MHC class I and/or an MHC class II molecule. In the case of class I MHC/peptide complexes, the binding peptides typically have a length of 8-10 amino acids, although longer or shorter peptides may be active. In the case of class II MHC/peptide complexes, the binding peptides typically have a length of 10-25 amino acids and in particular of 13-18 amino acids, although longer and shorter peptides may be active.

Fusion molecules of the invention and the nucleic acids coding therefor can generally be prepared by recombinant DNA techniques such as preparation of plasmid DNA, cleavage of DNA with restriction enzymes, ligation of DNA, transformation or transfection of a host, cultivation of the host and isolation and purification of the expressed fusion molecule. Such methods are known and described for example in Sambrook et al., Molecular Cloning (2nd edition, 1989).

DNA coding for the antigen can be obtained by isolating DNA from natural sources or by known synthetic methods such as the phosphate triester method; cf., for example, Oligonucleotide Synthesis, IRL Press (M.J. Gait, editor, 1984). Synthetic oligonucleotides can also be prepared with the aid of commercially available automatic oligonucleotide synthesizers.

The proportions of MHC molecules in the fusion molecules of the invention suitably correspond, in

relation to the amino acid sequence, to naturally occurring MHC molecules from humans, mice or other rodents or other mammals or are derivatives thereof.

DNA sources coding for MHC proteins are known, such as human lymphoblastoid cells. After isolation, the gene coding for the MHC molecule, or an interesting part thereof, can be amplified by polymerase chain reaction (PCR) or other known methods. Suitable PCR primers for amplifying the gene for the MHC peptide can attach restriction sites to the PCR product.

It is preferred according to the invention to prepare DNA constructs which comprise nucleic acid sequences coding for the leader sequence, the transmembrane region and the cytoplasmic region, and which comprise a restriction cleavage site between the leader sequence and the transmembrane region, so that essentially any nucleotide sequence coding for an interesting antigen can be incorporated into the construct.

In a preferred method for preparing fusion molecules of the invention, DNA sequences are disposed in such a way that the C-terminal end of the leader sequence is linked to the N-terminal end of the antigen, the C-terminal end of the antigen is linked to the N-terminal end of the transmembrane region, and the C-terminal end of the transmembrane region is linked to the N-terminal end of the cytoplasmic region. As discussed above, restriction cleavage sites are preferably incorporated between the end of the leader sequence and the start of the transmembrane region, so that essentially any nucleic acid which codes for an interesting antigen can be linked to the nucleic acid sequence for the transmembrane region.

An expressed fusion molecule of the invention may be isolated and purified in a manner known per se.

Typically, the culture medium will be centrifuged and the supernatant will then be purified by affinity or immunoaffinity methods comprising the use of monoclonal antibodies which bind to the expressed fusion molecule. The fusion molecule may also comprise a sequence which assists purification, e.g. a 6xHis tag.

The ability of a fusion molecule of the invention to modulate the activity of a T-cell receptor (including inactivation of T-cell responses) can easily be determined by an in vitro assay. Typically, T cells are provided for the assays by transformed T-cell lines, such as T-cell hybridomas or T cells which are isolated from a mammal such as a human or a rodent such as a mouse. Suitable T-cell hybridomas are freely available or can be prepared in a manner known per se. T cells can be isolated in a manner known per se from a mammal; cf., for example, Shimonkevitz, R. et al., 1983, J. Exp. Med. 158: 303.

A suitable assay for determining whether a fusion molecule of the invention is able to modulate the activity of T cells takes place as follows by steps 1-4 hereinafter. T cells suitably express a marker which can be assayed and indicates the T-cell activation or modulation of T-cell activity after activation. Thus, the mouse T-cell hybridoma DO11.10, which expresses interleukin-2 (IL-2) on activation, can be used. IL-2 concentrations can be measured in order to determine whether a specific presenting peptide is able to modulate the activity of this T-cell hybridoma. A suitable assay of this type is carried out by the following steps:

1. T cells are obtained for example from an interesting T-cell hybridoma or by isolation from a mammal.

2. The T cells are cultivated under conditions which permit proliferation.

3. The growing T cells are brought into contact with antigen-presenting cells which in turn have been brought into contact with a fusion molecule of the invention or with a nucleic acid coding therefor.

4. The T cells are assayed for a marker, e.g. IL-2 production is measured.

The T cells used in the assays are incubated under conditions suitable for proliferation. For example, a DO11.10 T-cell hybridoma is suitably incubated in complete medium (RPMI 1640, supplemented with 10% FBS, penicillin/streptomycin, L-glutamine and 5×10^{-5} M 2-mercaptoethanol) at about 37°C with 5% CO_2. Serial dilutions of the fusion molecule of the invention can be assayed. T-cell activation signals are provided by antigen-presenting cells which have been loaded with the suitable antigenic peptide.

As an alternative to measuring an expressed protein such as IL-2, it is possible to determine the modulation of T-cell activation suitably by changes in the proliferation of antigen-dependent T cells, as measured by known radiolabeling methods. For example, a labeled (such as tritiated) nucleotide can be introduced into an assay culture medium. The introduction of such a labeled nucleotide into the DNA serves as measurand for T-cell proliferation. This assay is unsuitable for T cells not requiring antigen presentation for growth, such as T-cell hybridomas. The assay is suitable for measuring the modulation of T-cell activation by fusion molecules in the case of untransformed T cells isolated from mammals.

The ability of a fusion molecule of the invention to

induce an immune response, including making it possible to vaccinate against a target disease, can be determined simply by an in vivo assay. For example, a fusion molecule of the invention or a nucleic acid coding therefor can be administered to a mammal such as a mouse, and blood samples be taken from the mammal at the time of the first administration and several times at periodic intervals thereafter (such as 1, 2, 5 and 8 weeks after administration of the fusion molecule or of the nucleic acid coding therefor). Serum is obtained from the blood samples and assayed for the appearance of antibodies resulting from the immunization. Antibody concentrations can be determined. In addition, T lymphocytes can be isolated from the blood or from lymphatic organs and be functionally assayed for reactivity to the antigen or epitopes derived from the antigen. All the readout systems known to the skilled worker, inter alia proliferation assay, cytokine secretion, cytotoxic activity, tetramer analysis, can be used in this connection.

Methods of the invention for inducing an immune response, including vaccination of a living creature against a target disease, can be used in combination with known methods for inducing an immune response. For example, a fusion molecule of the invention or a nucleic acid coding therefor can be administered to a living creature in an arrangement or combination with administration of a vaccine composition in order to enhance or prolong the desired effect of such a vaccine composition.

The term "derived" means according to the invention that a particular entity, in particular a particular sequence, is present in the object from which it is derived, in particular an organism or molecule. In the case of nucleic acid and amino acid sequences, especially particular sequence regions, "derived"

additionally means that the relevant nucleic acid or amino acid sequence is derived, consistent with the definitions hereinafter, from a nucleic acid or amino acid sequence which is present in the object. Thus, the expression "sequence or region derived from an MHC molecule" means that the sequence or region is present in an MHC molecule or is derived, consistent with the definitions hereinafter, from a sequence or region which is present in an MHC molecule.

A nucleic acid is according to the invention preferably deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). Nucleic acids include according to the invention genomic DNA, cDNA, mRNA, recombinantly prepared and chemically synthesized molecules. A nucleic acid may according to the invention be in the form of a molecule which is single stranded or double stranded and linear or closed covalently to form a circle.

A sequence derived from a nucleic acid sequence or the expression "sequence derived from a nucleic acid sequence" relates according to the invention to homologous sequences and derivatives of the former sequence.

Homologous nucleic acid sequences display according to the invention at least 40%, in particular at least 50%, at least 60%, at least 70%, at least 80%, at least 90% and preferably at least 95%, at least 98 or at least 99% identity of the nucleotides.

A nucleic acid is "homologous" to another nucleic acid in particular when the two sequences of the complementary strands are able to hybridize with one another and enter into a stable duplex, the hybridization preferably taking place under conditions which permit specific hybridization between polynucleotides (stringent conditions). Stringent

conditions are described for example in Molecular Cloning: A Laboratory Manual, J. Sambrook et al., editors, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989 or Current Protocols in Molecular Biology, F.M. Ausubel et al., editors, John Wiley & Sons, Inc., New York, and relate for example to hybridization at 65°C in hybridization buffer (3.5 × SSC, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 2.5 mM NaH_2PO_4 (pH 7), 0.5% SDS, 2 mM EDTA). SSC is 0.15 M sodium chloride/0.15 M sodium citrate, pH 7. After the hybridization, the membrane onto which the DNA has been transferred is for example washed in 2 × SSC at room temperature and then in 0.1-0.5 × SSC/0.1 × SDS at temperatures of up to 68°C.

"Derivative" of a nucleic acid means according to the invention that single or multiple nucleotide substitutions, deletions and/or additions are present in the nucleic acid. The term "derivative" also includes in addition chemical derivatization of a nucleic acid on a base, a sugar or phosphate of a nucleotide. The term "derivative" also includes nucleic acids which comprise non-naturally occurring nucleotides and nucleotide analogs.

The nucleic acids described by the invention are preferably isolated. The term "isolated nucleic acid" means according to the invention that the nucleic acid (i) has been amplified in vitro, for example by polymerase chain reaction (PCR), (ii) has been produced recombinantly by cloning, (iii) has been purified, for example by cleavage and fractionation by gel electrophoresis, or (iv) has been synthesized, for example by chemical synthesis. An isolated nucleic acid is a nucleic acid which is available for manipulation by recombinant DNA techniques.

Nucleic acids which code for fusion molecules can according to the invention be alone or in combination with other nucleic acids, especially heterologous nucleic acids. In preferred embodiments, a nucleic acid is functionally connected to expression control sequences or regulatory sequences which may be homologous or heterologous in relation to the nucleic acid. A coding sequence and a regulatory sequence are "functionally" connected together if they are linked together covalently in such a way that expression or transcription of the coding sequence is under the control or under the influence of the regulatory sequence. If the coding sequence is to be translated into a functional protein and where there is a functional connection of a regulatory sequence to the coding sequence, induction of the regulatory sequence leads to transcription of the coding sequence without the occurrence of a shift in reading frame in the coding sequence or of an inability of the coding sequence to be translated into the desired protein or peptide.

The term "expression control sequence" or "regulatory sequence" includes according to the invention promoters, enhancers and other control elements which control the expression of a gene. In particular embodiments of the invention, the expression control sequences can be regulated. The exact structure of regulatory sequences may vary species-dependently or cell type-dependently, but generally includes 5'-non-transcribed and 5'-non-translated sequences which are involved in initiating transcription and translation, respectively, such as TATA box, capping sequence, CAAT sequence and the like. In particular, 5'-non-transcribed regulatory sequences include a promoter region which includes a promoter sequence for transcriptional control of the functionally connected gene. Regulatory sequences may also include enhancer

sequences or activator sequences located upstream.

In a preferred embodiment, the nucleic acid is according to the invention a vector, where appropriate having a promoter which controls the expression of a nucleic acid, e.g. of a nucleic acid which codes for a fusion molecule of the invention. In a preferred embodiment, the promoter is a T7, T3 or SP6 promoter.

The term "vector" is used in this connection in its most general meaning and includes any of the intermediate vehicles for a nucleic acid which make it possible, for example, for the nucleic acid to be introduced into prokaryotic and/or into eukaryotic cells and, where appropriate, be integrated into a genome. Such vectors are preferably replicated and/or expressed in the cell. An intermediate vehicle may be adapted for example for use in electroporation, in microprojectile bombardment, in liposomal administration, in transfer with the aid of agrobacteria or in insertion via DNA or RNA viruses. Vectors include plasmids, phagemids, bacteriophages or viral genomes.

The nucleic acids which code for a fusion molecule of the invention can be employed for transfection of host cells. Nucleic acids mean in this connection both recombinant DNA and RNA. Recombinant RNA can be prepared by in vitro transcription from a DNA template. It can moreover be modified before application by stabilizing sequences, capping and polyadenylation.

The term "host cell" relates according to the invention to any cell which can be transformed or transfected with an exogenous nucleic acid. The term "host cells" includes according to the invention prokaryotic (e.g. E. coli) or eukaryotic (e.g. dendritic cells, B cells, CHO cells, COS cells, K562 cells, yeast cells and

insect cells). Mammalian cells are particularly preferred, such as cells from humans, mice, hamsters, pigs, goats and primates. The cells may be derived from a large number of tissue types and include primary cells and cell lines. Specific examples include keratinocytes, peripheral blood leukocytes, bone marrow stem cells and embryonic stem cells. In further embodiments, the host cell is an antigen-presenting cell, in particular a dendritic cell, a monocyte or macrophage. A nucleic acid may be present in the host cell in a single or in multiple copies and is, in one embodiment, expressed in the host cell.

The term "expression" is used according to the invention in its most general meaning and includes the production of RNA or of RNA and protein. It also includes partial expression of nucleic acids. In addition, the expression may be transient or stable. Preferred expression systems in mammalian cells include pcDNA3.1 and pRc/CMV (Invitrogen, Carlsbad, CA), which comprise a selectable marker such as a gene which confers resistance to G418 (and thus makes selection of stably transfected cell lines possible), and the enhancer-promoter sequences of cytomegalovirus (CMV).

A nucleic acid coding for a fusion molecule of the invention may also include a nucleic acid sequence which codes for an MHC molecule, preferably for an HLA molecule. The nucleic acid sequence which codes for an MHC molecule may be present on the same expression vector as the nucleic acid which codes for the fusion molecule, or the two nucleic acids may be present on different expression vectors. In the latter case, the two expression vectors can be cotransfected into a cell.

A sequence derived from an amino acid sequence or the expression "sequence derived from an amino acid

sequence" relates according to the invention to homologous sequences and derivatives of the former sequence.

Homologous amino acid sequences exhibit according to the invention at least 40%, in particular at least 50%, at least 60%, at least 70%, at least 80%, at least 90% and preferably at least 95%, at least 98 or at least 99% identity of the amino acid residues.

"Derivatives" of a protein or polypeptide or of an amino acid sequence in the sense of this invention include amino acid insertion variants, amino acid deletion variants and/or amino acid substitution variants.

Amino acid insertion variants include amino- and/or carboxy-terminal fusions, and insertions of single or multiple amino acids in a particular amino acid sequence. In amino acid sequence variants with an insertion, one or more amino acid residues are introduced into a predetermined site in an amino acid sequence, although random insertion with suitable screening of the resulting product is also possible. Amino acid deletion variants are characterized by deletion of one or more amino acids from the sequence. Amino acid substitution variants are distinguished by at least one residue in the sequence being deleted and another residue being inserted in its stead. The modifications are preferably present at positions in the amino acid sequence which are not conserved between homologous proteins or polypeptides. Amino acids are preferably replaced by others having similar properties, such as hydrophobicity, hydrophilicity, electronegativity, volume of the side chain and the like (conservative substitution). Conservative substitutions relate for example to replacement of one amino acid by another, with both amino acids being

listed in the same group hereinafter:

1. small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr (Pro, Gly)
2. negatively charged residues and their amides: Asn, Asp, Glu, Gln
3. positively charged residues: His, Arg, Lys
4. large aliphatic, nonpolar residues: Met, Leu, Ile, Val (Cys)
5. large aromatic residues: Phe, Tyr, Trp.

Three residues are put in parentheses because of their particular role in protein architecture. Gly is the only residue without a side chain and thus confers flexibility on the chain. Pro has an unusual geometry which greatly restricts the chain. Cys can form a disulfide bridge.

The amino acid variants described above can easily be prepared with the aid of known peptide synthesis techniques such as, for example, by solid phase synthesis (Merrifield, 1964) and similar methods or by recombinant DNA manipulation. Techniques for introducing substitution mutations at predetermined sites in DNA which has a known or partially known sequence are well known and include, for example, M13 mutagenesis. Manipulation of DNA sequences to prepare proteins having substitutions, insertions or deletions and the general recombinant methods for expression of proteins for example in a biological system (such as mammalian, insect, plant and viral systems) are described in detail for example in Sambrook et al. (1989).

"Derivatives" of proteins or polypeptides also include according to the invention single or multiple substitutions, deletions and/or additions of any molecules which are associated with the protein or

polypeptide, such as carbohydrates, lipids and/or proteins or polypeptides.

In one embodiment, "derivatives" of proteins or polypeptides include those modified analogs resulting from glycosylation, acetylation, phosphorylation, amidation, palmitoylation, myristoylation, isoprenylation, lipidation, alkylation, derivatization, introduction of protective/blocking groups, proteolytic cleavage or binding to an antibody or to another cellular ligand. Derivatives of proteins or polypeptides may also be prepared by other methods such as, for example, by chemical cleavage with cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, $NaBH_2$, acetylation, formylation, oxidation, reduction or by metabolic synthesis in the presence of tunicamycin.

The term "derivative" also extends to all functional chemical equivalents of proteins or polypeptides.

The derivatives, described above, of proteins and polypeptides are encompassed according to the invention by the term "fusion molecule", even if no express reference is made thereto.

The pharmaceutical compositions described according to the invention can be employed therapeutically for the treatment of a pre-existing disease or prophylactically as vaccines for immunization.

The term "vaccine" relates according to the invention to an antigenic preparation which comprises for example a protein, a peptide, a nucleic acid or a polysaccharide, and which is administered to a recipient in order to stimulate its humoral and/or cellular immune system against one or more antigens which are present in the vaccine preparation. The terms "vaccination" or "immunization" relate to the process

of administering a vaccine and of stimulating an immune response against an antigen. The term "immune response" relates to the activities of the immune system, including activation and proliferation of specific cytotoxic T cells after contact with an antigen.

Animal models can be employed for testing an immunizing effect, e.g. against cancer on use of a tumor-associated antigen as antigen. It is moreover possible for example, for human cancer cells to be introduced into a mouse to create a tumor, and for a nucleic acid of the invention, which codes for a fusion molecule of the invention comprising the tumor-associated antigen, to be administered. The effect on the cancer cells (for example reduction in tumor size) can be measured as criterion for the efficacy of an immunization by the nucleic acid.

As part of the composition for immunization, one or more fusion molecules are administered with one or more adjuvants to induce an immune response or increase an immune response. An adjuvant is a substance which is incorporated into an antigen or is administered together therewith and enhances the immune response. Adjuvants are able to enhance the immune response by providing an antigen reservoir (extracellularly or in macrophages), activating macrophages and stimulating certain lymphocytes. Adjuvants are known and include in a nonrestrictive manner monophosphoryl-lipid A (MPL, SmithKline Beecham), saponins such as QS21 (SmithKline Beecham), DQS21 (SmithKline Beecham; WO 96/33739), QS7, QS17, QS18 and QS-L1 (So et al., Mol. Cells 7:178-186, 1997), incomplete Freund's adjuvant, complete Freund's adjuvant, vitamin E, Montanide, alum, CpG oligonucleotides (cf. Krieg et al., Nature 374:546-9, 1995) and various water-in-oil emulsions which are prepared from biodegradable oils such as squalene and/or tocopherol. The fusion molecules are preferably

administered in a mixture with DQS21/MPL. The ratio of DQS21 to MPL is typically about 1:10 to 10:1, preferably about 1:5 to 5:1 and in particular about 1:1. In a vaccine formulation for administration to humans, DQS21 and MPL are typically present in a range from about 1 μg to about 100 μg.

Other substances which stimulate an immune response in the patient may also be administered. For example, cytokines can be used for a vaccination because of their regulatory properties on lymphocytes. Such cytokines include for example interleukin-12 (IL-12) which has been shown to enhance the protective effects of vaccines (cf. Science 268:1432-1434, 1995), GM-CSF and IL-18.

The method of the invention for inducing an immune response in a mammal generally comprises the administration of an effective amount of a fusion molecule of the invention and/or of a nucleic acid coding therefor, in particular in the form of a vector. DNA or RNA which codes for a fusion molecule of the invention is preferably administered to a mammal together with a DNA sequence which codes for a T cell-costimulating factor, such as a gene coding for B7-1 or B7-2.

The expression "T cell-costimulating factor" relates herein to a molecule, in particular a peptide, which is able to provide a costimulating signal and thus enhances an immune response, in particular activates the proliferation of T cells in the presence of one or more fusion molecules of the invention. Such an activation of T-cell proliferation can be determined by generally known assays.

These factors include costimulating molecules which are provided in the form of proteins or nucleic acids.

Examples of such costimulating molecules are B7-1 and B7-2 (CD80 and CD86, respectively) which are expressed on dendritic cells (DC) and interact with the CD28 molecule expressed on T cells. This interaction provides a costimulation (signal 2) for an antigen/MHC/TCR-stimulated (signal 1) T cell, thus enhancing the proliferation of the T cell and the effector function. B7 also interacts with CTLA4 (CD152) on T cells and investigations including CTLA4 ligands and B7 ligands show that the B7-CTLA4 interaction can enhance an antitumor immunity and CTL proliferation (Zheng, P. et al., Proc. Natl. Acad. Sci. USA 95(11):6284-6289 (1998)).

B7 is typically not expressed on tumor cells, so that they are not effective antigen-presenting cells (APCs) for T cells. Induction of B7 expression would make it possible for tumor cells more effectively to stimulate proliferation of cytotoxic T lymphocytes and an effector function. Costimulation by a B7/IL-6/IL-12 combination showed an induction of the IFN-gamma and Th1 cytokine profile in a T cell population, leading to a further enhancement of T-cell activity (Gajewski et al., J. Immunol. 154:5637-5648 (1995)).

Complete activation of cytotoxic T lymphocytes and a complete effector function requires cooperation of T-helper cells through the interaction between the CD40 ligand on the T-helper cells and the CD40 molecule which is expressed by dendritic cells (Ridge et al., Nature 393:474 (1998), Bennett et al., Nature 393:478 (1998), Schönberger et al., Nature 393:480 (1998)). The mechanism of this costimulating signal probably relates to increasing the B7 and associated IL-6/IL-12 production by the dendritic cells (antigen-presenting cells). The CD40-CD40L interaction thus complements the interactions of signal 1 (antigen/MHC-TCR) and signal 2 (B7-CD28).

The invention provides for administration of nucleic acids, polypeptides or proteins and/or cells. Administration of DNA and RNA is preferred.

It was possible to show in the experiments that, compared with the unmodified antigen, according to the invention a 100-fold lower dose of the vaccine is sufficient to induce equivalent or stronger immune responses. One problem on direct injection of nucleic acid vaccines is that the dose necessary to induce immune responses is very high. In the case of DNA vaccines, the reason is presumably mainly based on the fact that only a fraction of the cells take up injected DNA into the nucleus. In the case of RNA vaccines, the problem is presumably that in particular injected RNA is very rapidly degraded by RNAses.

It is to be expected on use of the vaccines modified according to the invention that greatly increased immune responses will be obtained on direct injection of nucleic acids, in particular RNA, compared with unmodified nucleic acids.

In a preferred embodiment, a viral vector for administering a nucleic acid which codes for a fusion molecule of the invention is selected from the group consisting of adenoviruses, adeno-associated viruses, poxviruses, including vacciniavirus and attenuated poxviruses, Semliki forest virus, retroviruses, Sindbis virus and Ty virus-like particles. Adenoviruses and retroviruses are particularly preferred. The retroviruses are normally replication-deficient (i.e. they are unable to produce infectious particles).

Various methods can be employed according to the invention to introduce nucleic acids into cells in vitro or in vivo. Such methods include transfection of

nucleic acid-calcium phosphate precipitates, transfection of nucleic acids associated with DEAE, transfection or infection with the above viruses carrying the nucleic acids of interest, liposome-mediated transfection and the like. In particular embodiments, guiding of the nucleic acid to particular cells is preferred. In such embodiments, a carrier employed for administering a nucleic acid to a cell (e.g. a retrovirus or a liposome) may have a bound targeting molecule. For example, a molecule such as an antibody which is specific for a surface membrane protein on the target cell, or a ligand for a receptor on the target cell, can be incorporated into the nucleic acid carrier or bound thereto. If administration of a nucleic acid by liposomes is desired, it is possible to incorporate proteins which bind to a surface membrane protein which is associated with endocytosis into the liposome formulation in order to make targeting and/or uptake possible. Such proteins include capsid proteins or fragments thereof, which are specific for a particular cell type, antibodies against proteins which are internalized, proteins which target for an intracellular site, and the like.

The nucleic acids are preferably administered together with stabilizing substances such as RNA-stabilizing substances.

In one embodiment, the nucleic acids are administered by ex vivo methods, i.e. by removing cells from a patient, genetically modifying the cells, and reintroducing the modified cells into the patient. This generally includes the introduction of a functional copy of a gene into the cells of a patient in vitro and returning the genetically modified cells to the patient. The functional copy of the gene is under the functional control of regulatory elements which permit expression of the gene in the genetically modified

cells. Transfection and transduction methods are known to the skilled worker. The invention also provides for administration of nucleic acids in vivo through the use of vectors such as viruses and targeted liposomes.

Administration of polypeptides and peptides can take place in a manner known per se.

The term "patient", "individual" or "living creature" means according to the invention a human, non-human primate or another animal, in particular mammal such as cow, horse, pig, sheep, goat, dog, cat, birds such as chicken or rodent such as mouse and rat. In a particularly preferred embodiment, the patient, the individual or the living creature is a human.

The therapeutic compositions of the invention can be administered in pharmaceutically acceptable preparations. Such preparations can comprise usually pharmaceutically acceptable concentrations of salts, buffering substances, preservatives, carriers, supplementary immunity-increasing substances such as adjuvants (e.g. CpG oligonucleotides) and cytokines and, where appropriate, other therapeutic agents.

The therapeutic agents of the invention can be administered in any conventional way, including by injection or by infusion. The administration can take place, for example, orally, intravenously, intraperitoneally, intramuscularly, subcutaneously, intracutaneously, transdermally, intralymphatically, preferably by injection into lymph nodes, especially inguinal lymph nodes, lymphatic vessels and/or into the spleen.

The compositions of the invention are administered in effective amounts. An "effective amount" relates to the amount which, alone or together with further doses,

achieves a desired response or a desired effect. In the case of treatment of a particular disease or of a particular condition, the desired response relates to inhibition of the progress of the disease. This includes slowing down the progression of the disease and in particular stopping the progression of the disease. The desired response on treatment of a disease or of a condition may also be delaying the onset or preventing the onset of the disease or of the condition.

An effective amount of a composition of the invention depends on the condition to be treated, the severity of the disease, the individual patient's parameters, including age, physiological condition, height and weight, the duration of the treatment, the nature of a concomitant therapy (if present), the specific administration route and similar factors.

The pharmaceutical compositions of the invention are preferably sterile and comprise an effective amount of the therapeutically active substance to generate the desired response or the desired effect.

The doses of the compositions of the invention which are administered may depend on various parameters such as the mode of administration, the patient's condition, the desired administration period etc. In the case where a patient's response is inadequate with an initial dose, it is possible to employ higher doses (or effectively higher doses which are achieved by a different, more localized administration route)..

In general, doses of from 1 ng to 1 mg, preferably from 10 ng to 100 µg, of the tumor-associated antigen are formulated and administered for a treatment or for generating or enhancing an immune response. If it is desired to administer nucleic acids (DNA and RNA),

doses of from 1 ng to 0.1 mg are formulated and administered.

The pharmaceutical compositions of the invention are generally administered in pharmaceutically acceptable amounts and in pharmaceutically acceptable compositions. The term "pharmaceutically acceptable" relates to a non-toxic material which does not interact with the effect of the active ingredient of the pharmaceutical composition. Such preparations may usually comprise salts, buffering substances, preservatives, carriers and, where appropriate, other therapeutic agents. When used in medicine, the salts should be pharmaceutically acceptable. Non-pharmaceutically acceptable salts can, however, be used to prepare pharmaceutically acceptable salts thereof and are encompassed by the invention. Such pharmacologically and pharmaceutically acceptable salts include in a non-limiting manner those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic acids and the like. Pharmaceutically acceptable salts can also be prepared as alkali metal or alkaline earth metal salts such as sodium, potassium or calcium salts.

A pharmaceutical composition of the invention may comprise a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier" relates according to the invention to one or more compatible solid or liquid fillers, diluents or capsule substances which are suitable for administration to a human. The term "carrier" relates to an organic or inorganic ingredient, natural or synthetic in nature, in which the active ingredient is combined in order to facilitate use. The ingredients of the pharmaceutical composition of the invention are usually such that no interaction which substantially impairs the desired

pharmaceutical activity occurs.

The carriers are preferably sterile liquids such as water or oils, including those derived from petroleum, animals or plants, or being of synthetic origin, such as, for example, peanut oil, soybean oil, mineral oil, sesame oil, sunflower oil and the like. Saline solutions and aqueous dextrose and glycerol solutions can also be used as aqueous carriers.

Examples of excipients and carriers are acrylic and methacrylic derivatives, alginic acid, sorbic acid derivatives such as α-octadecyl-ω-hydroxypoly(oxyethylene)-5-sorbic acid, amino acids and their derivatives, especially amine compounds such as choline, lecithin and phosphatidylcholine, gum arabic, aromas, ascorbic acid, carbonates such as, for example, sodium, potassium, magnesium and calcium carbonates and bicarbonates, hydrogen phosphates and phosphates of sodium, potassium, calcium and magnesium, carmellose sodium, dimethicone, colors, flavorings, buffering substances, preservatives, thickeners, plasticizers, gelatin, glucose syrups, malt, colloidal silicon dioxide, hydromellose, benzoates, especially sodium and potassium benzoates, macrogol, skim milk powder, magnesium oxide, fatty acids and their derivatives and salts such as stearic acid and stearates, especially magnesium and calcium stearates, fatty acid esters and mono- and diglycerides of edible fatty acids, natural and synthetic waxes such as beeswax, yellow wax and montan glycol wax, chlorides, especially sodium chloride, polyvidone, polyethylene glycols, polyvinylpyrrolidone, povidone, oils such as castor oil, soybean oil, coconut oil, palm kernel oil, sugars and sugar derivatives, especially mono- and disaccharides such as glucose, fructose, mannose, galactose, lactose, maltose, xylose, sucrose, dextrose and cellulose and their derivatives, shellac, starch and starch

derivatives, especially corn starch, tallow, talc, titanium dioxide, tartaric acid, sugar alcohols such as glycerol, mannitol, sorbitol and xylitol and their derivatives, glycol, ethanol and mixtures thereof.

The pharmaceutical compositions may preferably also comprise in addition wetting agents, emulsifiers and/or pH-buffering agents.

In a further embodiment, the pharmaceutical compositions may comprise an absorption enhancer. These absorption enhancers may if desired replace an equimolar amount of the carrier in the composition. Examples of such absorption enhancers include in a non-limiting manner eucalyptol, N,N-diethyl-m-toluamide, polyoxyalkylene alcohols (such as propylene glycol and polyethylene glycol), N-methyl-2-pyrrolidone, isopropyl myristate, dimethylformamide (DMF), dimethyl sulfoxide (DMSO), dimethylacetamide (DMA), urea, diethanolamine, triethanolamine and the like (see, for example, Percutaneous Penetration Enhancers, edited by Smith et al. (CRC Press, 1995)). The amount of absorption enhancer in the composition may depend on the desired effects to be achieved.

A protease inhibitor can be incorporated into the composition of the invention in order to prevent degradation of a peptide or protein agent and thus to increase the bioavailability. Examples of protease inhibitors include in a non-limiting manner aprotinin, leupepsin, pepstatin, α2-macroglobulin and trypsin inhibitor. These inhibitors can be used alone or in combination.

The pharmaceutical compositions of the invention can be provided with one or more coatings. The solid oral dosage forms are preferably provided with a coating resistant to gastric juice or are in the form of a

hardened soft gelatin capsule resistant to gastric juice.

The pharmaceutical compositions of the invention may comprise suitable buffering substances such as acetic acid in a salt, citric acid in a salt, boric acid in a salt and phosphoric acid in a salt.

The pharmaceutical compositions may also comprise where appropriate suitable preservatives such as benzalkonium chloride, chlorobutanol, parabens and thimerosal.

The pharmaceutical compositions are usually presented in a unit dose form and can be produced in a manner known per se. Pharmaceutical compositions of the invention may be for example in the form of capsules, tablets, lozenges, solutions, suspensions, syrups, elixirs or as emulsion.

Compositions suitable for parenteral administration usually comprise a sterile aqueous or nonaqueous preparation of the active agent, which is preferably isotonic with the recipient's blood. Examples of suitable carriers and solvents are Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are usually employed as dissolving or suspending medium.

The present invention is described in detail by the following examples and figures which serve exclusively for illustration and are not to be understood as limiting. Further embodiments which do not go beyond the bounds of the invention and the scope of the annexed claims are accessible to the skilled worker on the basis of the description and the examples.

Brief description of the drawings:

Figure 1: Diagrammatic representation of a fusion protein of the invention. The fusion protein consists of an N-terminally placed secretion signal, of a C-terminally located transmembrane and cytoplasmic domain of a histocompatibility antigen, and of an integrated complete or partial sequence of an antigen.

Figure 2: Diagrammatic representation of the cassettes for expression of fusion proteins. SP: signal peptide; MCS: multiple cloning site; TM: transmembrane domain; MHC tail: cytoplasmic tail of an MHC molecule; antigen: sequence coding for an antigen against which immune responses are to be induced.

Figure 3: Testing of the effect of various RNA doses on the frequency of antigen-specific CD4+ T lymphocytes. 1×10^6 purified CD4+ lymphocytes were cocultivated for 1 week with 2×10^5 DC which had been transfected with RNA in the stated amounts (0.1-10 µg RNA) by electroporation. On day 7 after stimulation, an ELISPOT was carried out under standard conditions to detect interferon-γ-secreting T lymphocytes. The antigen-presenting cells used were DC from the same donor which had been loaded with overlapping pp65 peptides (1.75 µg/ml) or an irrelevant control peptide. For the test, 3×10^4 effectors were coincubated with 2×10^4 DC for 16 h. After standard development, the number of IFN-gamma-secreting T lymphocytes was determined by means of a software-based video analysis. Compared with the CMVpp65standard RNA, there is seen to be a massive expansion of CD4+ lymphocytes both by the CMVpp65-TM1 construct and by the CMVpp65-TM2 construct.

Figure 4: Testing of the effect of various RNA doses on the frequency of interferon-gamma-secreting CD8+ T lymphocytes. 1×10^6 purified CD8+ lymphocytes were cocultivated for 1 week with 2×10^5 DC which had been transfected with RNA in the stated amounts (0.1-10 µg

RNA) by electroporation. On day 7, a standard ELISPOT was carried out to detect IFN-gamma-secreting T lymphocytes against DC of the same donor which had been loaded with overlapping pp65 peptides (1.75 μg/ml) or an irrelevant control peptide. 3×10^4 effectors were coincubated with 2×10^4 DC for 16 h. After standard development, the number of IFN-gamma-secreting T lymphocytes was determined by means of a software-based video analysis. There was seen to be a massive expansion of CD8+ lymphocytes by the CMVpp65-TM1 construct and the CMVpp65-TM2 construct. Even on use of 100× lower doses (0.1 μg RNA), the frequency of the pp65-specific CD8+ lymphocytes was still above the background after stimulation by DC transfected with NYESO-RNA (data not shown). Stimulation by the CMVpp65standard construct showed an expansion of pp65-specific lymphocytes above the background level only with 2.5 μg and above.

Figure 5: Dose/effect profile for the expansion capacity of various immunogens on antigen-specific lymphocytes. The immunogens modified according to the invention exhibit a distinctly increased potency (>100×) and a higher maximum effect.

Figure 6: Comparative test of the effect of immunogens modified according to the invention and standard immunogens on the generation of cytotoxic immune responses. 1×10^6 purified CD8+ lymphocytes were cocultivated for 1 week with 2×10^5 DC which had been transfected with 10 μg of RNA by electroporation. On day 7, a standard cytochrome cytotoxicity assay against DC of the same donor which had been loaded with various concentrations of overlapping pp65 peptides or an irrelevant control peptide was carried out. 15×10^4 effectors were coincubated with 0.5×10^4 DC for 4 h. After measurement of the supernatant in a counter, the specific lysis was calculated according to the formula:

There was seen to be extensive lysis by CD8+ lymphocytes which had been stimulated with CMVpp65-TM1 and CMVpp65-TM2 constructs, which was above the value for the control peptide as far as a concentration of 10 nM of the pp65 peptide mixture (data not shown). CD8+ lymphocytes were likewise expanded by the pp65 peptide mixture and showed a marked specific lysis, but did not reach the level of CMVpp65-TM1 and -TM2. Only a weak stimulation of pp65-specific cytotoxic T cells was achievable by the CMVpp65standard construct.

Figure 7: Diagrammatic representation of the cassettes for expressing fusion proteins. CS: cloning site; TM: transmembrane domain; SNARE: SNARE protein or motif; antigen: sequence coding for an antigen against which immune responses are to be induced.

Figure 8: Sequences used in the examples HLA class I TM-CM: transmembrane-cytoplasmic region of an HLA class I molecule; HLA class II TM-CM: transmembrane-cytoplasmic region of an HLA class II molecule.

Figure 9: Sequences of transmembrane-cytoplasmic regions and cytoplasmic regions of MHC molecules. The sequences show the transmembrane-cytoplasmic region or only the cytoplasmic region of various HLA molecules. The transmembrane region is underlined and bold.

Figure 10: Sequences of SNARE proteins. These sequences are suitable for constructing the SNARE-antigen fusion molecules (N-SNARE-antigen) of the invention.

Figure 11: Stimulation of naive CD8+ T lymphocytes by fusion constructs of the invention. In microtiter plates, 1×10^5 CD8+ lymphocytes per well were stimulated against 2×10^4 DC which were transfected with 20 µg of CMVpp65-TM1 or control RNA. The medium was supplemented with IL-6 (1000 U/ml) and IL-12 (10 ng/ml). On day +7

and +14, thawed transfected DC (2×10^4/well) were used for restimulation, the medium containing IL-2 (10 U/ml) and IL-7 (5 ng/ml). On day +21, all the populations were assayed in an ELISPOT against control peptides (1.75 µg/ml) and against pp65-overlapping peptides (1.75 µg/ml). Two of the populations stimulated against CMVpp65-TM1 (Pop.1, Pop.2) showed a marked pp65 reactivity.

Examples:

Example 1: Preparation of the modified vaccines

To prepare the modified vaccines, firstly a cassette which permits expression of fusion genes was prepared in an expression vector which permits transcription of RNA. For this purpose, initially the nucleic acid which codes for a signal peptide of an HLA molecule was amplified from human lymphocytes, and the fragment was cloned as cDNA into a vector (SEQ ID NO: 1 and 2). The cloning was carried out in such a way that various restriction enzyme cleavage sites were located behind the cDNA of the signal peptide, and further fragments can be cloned in-frame in the expression cassette. The selected vectors were plasmids which permit in vitro expression of RNA via a 5'-located RNA polymerase promoter T3, T7 or SP6. The next fragment cloned into this vector was a cDNA which encodes a transmembrane domain and the cytoplasmic domain of an HLA class I (SEQ ID NO: 3 and 4) or class II (SEQ ID NO: 5 and 6) molecule, including stop codon. The cloning was carried out in such a way that the resulting plasmid still has restriction enzyme cleavage sites for cloning antigens between the two fragments (SEQ ID NO: 7 and 8 and Figure 1). The sequence (SEQ ID NO: 9 and 10) coding for the human cytomegalovirus phosphoprotein 65 (pp65) was cloned into these expression cassettes as model antigen in such a way that a continuous ORF composed of

HLA signal sequence, pp65 and HLA transmembrane and cytoplasmic domain (SEQ ID NO: 11 and 12) resulted. A vector which comprised the pp65 sequence with a stop codon in the same initial vector without said fragments was prepared for control experiments. The following nucleic acids were used for further experiments:

CMVpp65standard: unmodified CMVpp65 sequence, standard immunogen

CMVpp65-TM1: fusion nucleic acid composed of the following fragments: HLA class I secretion signal, pp65 ORF and HLA class I transmembrane and cytoplasmic domain (modified immunogen).

CMVpp65-TM2: fusion nucleic acid composed of the following fragments: HLA class I secretion signal, pp65 ORF and HLA class II transmembrane and cytoplasmic domain (modified immunogen).

Example 2: Testing of the modified vaccines

The three nucleic acids (CMVpp65standard, CMVpp65TM1, CMVpp65TM2) were employed as immunogen in stimulation tests with autologous DCs from antigen-positive donors. In order to test CD4 and CD8 immune responses separately, purified CD4+ and CD8+ lymphocytes were used. The readout employed was the enzyme-linked immunospot assay (ELISPOT), which is acknowledged to be the standard assay for quantifying IFN-λ-secreting T cells. A standard chromium release assay was used to assay the effector function of CD8+ T lymphocytes. Autologous monocytes or DCs were transfected with pp65 RNA, CMVpp65-TM1 and CMVpp65-TM2 immunogens. DCs were loaded with overlapping peptides for pp65 and with control peptide as maximum stimulation control. The DCs treated in this way were coincubated with CD4+ or CD8+ lymphocytes overnight or for 7 days. The readout took

place against autologous monocytes or DCs which had been pulsed with pp65 overlapping peptides or with a CMV fibroblast lysate. The investigation of CD4+ immune responses surprisingly revealed that both modified immunogens (CMVpp65-TM1 and CMVpp65-TM2) not only induced an enhanced immune response to the CMVpp65standard immunogen, but also induced a maximum level of antigen-specified IFN-gamma secretion in CD4+ lymphocytes (Figure 3). The percentage of antigen-specific CD4+ cells after stimulation by the modified pp65 constructs was moreover equal to or even higher than after stimulation with pp65 overlapping peptides. As expected, the CMVpp65standard immunogen showed no relevant stimulation of CD4+ lymphocytes.

An even more surprising result emerged on investigation of CD8 immune responses after stimulation with the immunogens. It was possible to show that the use of the modified expression cassettes for stimulating CD8+ lymphocytes likewise led to a proportion of specifically IFN-λ-secreting cells which is comparable to that after stimulation with pp65 overlapping peptides. Surprisingly, the modified RNA constructs were far superior to the unmodified CMVpp65standard immunogens in this case too (Figures 4 and 5). The results in the cytotoxicity assay showed that both modifications led to a not previously described drastic increase in cytotoxicity compared with CMVpp65standard RNA (Figure 6). In this case too there was surprisingly seen to be a superiority of the modified immunogens over the overlapping pp65 peptides.

Example 3: Stimulation of naive CD8+ T lymphocytes by HLA fusion antigens

In order to attest the possibility of priming and subsequent expansion of naive CD8+ lymphocytes by the fusion constructs of the invention, dendritic cells of

a CMV-negative donor were transfected with RNA of the unmodified CMVpp65 or with CMVpp65-TM1 RNA or with a control RNA (NY-Eso-1). The transfected dendritic cells were employed to stimulate autologous CD8+ lymphocytes. 2 restimulations were carried out with frozen transfected dendritic cells at weekly intervals. For the readout, on day +21 after the first stimulation, all cell populations were assayed in an IFNγ ELISpot assay against autologous dendritic cells which were loaded either with pp65 overlapping peptides or, as control, with irrelevant overlapping peptides. It was found in this case that pp65-reactive CD8+ T lymphocyte populations were generated by stimulation with CMVpp65-TM1 RNA in two cases (Figure 11). Stimulations with the dendritic cells transfected with the unmodified CMVpp65 RNA or with control RNA by contrast showed no significant pp65 reactivity.

Example 4: Use of HLA fusion antigens for stimulating tumor cell-reactive T lymphocytes

In order to be able to expand CD8+ and CD4+ T lymphocytes against defined tumor antigens, the following antigen sequences were cloned as inserts into fusion constructs of the invention: the tumor antigen TPTE (Koslowski et al., 2004, PMID 15342378), the tumor antigen PRAME (Ikeda et al., 1997, PMID 9047241) in variant 1 (SEQ ID NO: 64), the tumor antigen WT1 as variant C (SEQ ID NO: 65) and the tumor antigen p53 (SEQ ID NO: 66). For the functional validation, human dendritic cells of an HLA* A 0201-positive donor were transfected either with WT1-HLA-TM1-RNA, with unmodified WT1-RNA or irrelevant control RNA and used as target cells. After coincubation with WT1-reactive CD8+ T-cell clones for 8 or 16 hours, IFNγ was quantified in the supernatant. It was seen that secretion was a factor of 6-9 higher after coincubation with WT1-HLA-TM1 transfected dendritic cells by

comparison with coincubation after transfection with unmodified WT1.

In a series of experiments, the following results were achieved in summary and confirmed several times:

- The modified immunogens lead to a distinctly enhanced stimulation and expansion of antigen-specific CD4+ lymphocytes (increased proliferation of CD4+ lymphocytes)

- The modified immunogens lead to a distinctly enhanced stimulation and expansion of antigen-specific CD8+ lymphocytes (increased proliferation of CD8+ lymphocytes)

- The modified immunogens lead to a distinctly enhanced cytokine release from antigen-specific CD4+ lymphocytes and CD8+ lymphocytes (increased cytokine release = increased activation)

- The modified immunogens lead to a distinctly enhanced cytotoxic reactivity of antigen-specific CD8+ lymphocytes (increased cytotoxic effect)

- The modified immunogens are 100x more potent in relation to the expansion of antigen-specific CD8+ lymphocytes

- The modified immunogens have, even at a 100x lower dose, a stronger effect on the expansion of antigen-specific CD4+ lymphocytes than standard immunogens

In summary, therefore, it can be said that the modifications according to the invention of an antigen result in a more than 100-fold increased potency (leftward shift in the dose-effect curve) and a drastically increased biological activity. Compared

with the unmodified antigen sequences customary to date, it is possible to generate an immunogen which has a quantitatively and qualitatively greater efficacy as vaccine.

An important result of the invention is that antigen-specific CD4+ and CD8+ lymphocytes are optimally stimulated and expanded simultaneously. Stimulation of CD8+ and CD4+ lymphocytes is crucially important for the efficacy in particular of therapeutic vaccines.